The Effect of Ovary Storage and In Vitro Maturation on mRNA Levels in Bovine Oocytes; A Possible Impact of Maternal ATP1A1 on Blastocyst Development in Slaughterhouse-derived Oocytes

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Abstract. Since BSE testing of slaughtered cattle is obligatory in Japan, storage of ovaries at 15–20 C overnight in phosphate buffered saline has become a routine protocol in in vitro production (IVP) of cattle embryos. Ovary storage is known to reduce developmental competence of oocytes; however, its effects on oocyte gene expression have not been clarified yet. This study compared oocytes collected from stored slaughterhouse-derived ovaries with those collected by Ovum Pick-Up (OPU) in terms of the expression of 20 selected genes to determine if ovary storage affects cellular processes at the molecular level. Expression of mRNA in oocytes was assayed before and after in vitro maturation (IVM) by real-time quantitative PCR. Maternal mRNA levels of genes were investigated in 2-cell stage embryos obtained from slaughterhouse oocytes to assess their roles for blastocyst formation. In immature OPU oocytes, genes related to metabolism (GAPDH), transporters (GLUT8, ATP1A1) and stress resistance protein (HSP70) showed significantly higher expression compared with oocytes derived from stored ovaries. During IVM, the expression of GDF9, GLUT8, CTNNB1 and PMSB1 was significantly decreased irrespective of oocyte source. Two-cell stage embryos cleaving at 22–25 h after in vitro fertilization (IVF) showed a significantly higher blastocyst formation rate and ATP1A1 gene expression level compared with those cleaving at 27–30 h after IVF. Our results reveal that storage of ovaries alters mRNA levels in oocytes. Correlation of Na/K ATPase ATP1A1 expression in IVP embryos at the 2-cell and 8-cell stages with their developmental ability to the blastocyst stage may suggest the importance of maternal mRNA of this gene during blastulation in embryos derived from slaughterhouse oocytes.

Key words: Bovine, mRNA expression, Oocyte, OPU, Ovary storage

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In recent decades, in vitro production (IVP) using in vitro matured (IVM) follicular oocytes collected from slaughtered animals has become a routine system for mass production of bovine embryos [1]. In IVP systems, oocyte competence basically determines the success in generating blastocysts [2]. Oocyte competence is known to be affected by several factors such as follicular status [2, 3] and environmental conditions during IVM [4, 5].

Transportation of slaughterhouse ovaries to the laboratory requires storage to some extent. It has been revealed that long-term storage at low temperatures such as 20 C is more favorable to competence of oocytes collected from the stored ovaries than storage at nearly physiological temperatures [6]. Since BSE testing of slaughtered cattle is obligatory in Japan, overnight storage of ovaries at 15–20 C until confirmation of negative test results has become a routine protocol in cattle IVP systems. Treatment of oocytes under such unphysiological conditions suggests alterations in cellular processes and consequently in meiotic and developmental competence; however, there are inconsistent reports regarding the harmfulness of long-term cold storage of ovaries on oocyte competence. Some papers reported similar meiotic and developmental competence of bovine oocytes obtained from fresh or stored ovaries [7], whereas others demonstrated reduced developmental competence due to ovary storage [6].

The developmental competence of oocytes is generally believed to be determined by stored substrates such as maternal mRNA, which accumulates in the cytoplasm of follicular oocytes during the growth stage [8]. Since fully grown bovine oocytes remain transcriptionally inactive [9] until the embryonic genome activation at the 8-cell stage [10], protein synthesis required for oocyte maturation, fertilization and early embryo development is thought to be mainly conducted by stored maternal mRNA. Therefore, the abundance of maternal mRNA in oocytes is believed to determine their developmental competence. Accordingly, the mRNA levels of some genes in bovine oocytes and cleavage stage embryos were found to correlate with developmental and meiotic competence [11–14]. It seems possible therefore that stresses during ovary stor-
age affect cellular processes at the molecular level. However, the effects of ovary storage on mRNA levels in oocytes from stored ovaries and further developmental competence remain unknown. Furthermore, several genes have been found to be differentially expressed between in vivo matured and IVM bovine oocytes [15, 16], suggesting that inadequate culture conditions may also alter mRNA expression in IVM oocytes.

The aim of the present study was to reveal the effects ovary storage and IVM on basic cellular processes on the molecular level and their possible contribution to embryo developmental competence to the blastocyst stage after IVF. Maternal mRNA levels in oocytes collected from slaughterhouse-derived stored ovaries or by OPU were studied before and after IVM. Twenty genes that have already been proven to be developmentally important and/or affected by the IVP procedure in oocytes/embryos in previous studies were investigated. Also, the levels of maternal mRNA of these genes at the 2-cell stage and 8-cell stage of IVP embryos with high and low developmental competence were compared to reveal their importance for subsequent embryo development to the blastocyst stage.

**Materials and Methods**

**Oocyte collection from slaughtered cows**

Collection and IVM of bovine follicular oocytes were performed as described previously by Imai et al. [17]. Ovaries at various phases of the cycle from approximately 28–32-month-old Holstein-Friesian cows of unknown body condition were collected at a local slaughterhouse, transported to the laboratory and then washed and stored in phosphate buffered saline (PBS) supplemented with 50 µg/ml gentamicin (Sigma-Aldrich, St. Louis, MO, USA) at 15 C for approximately 15 h. Cumulus–oocyte complexes (COCs) were aspirated from small follicles (3–6 mm in diameter) using a 5-ml syringe with a 19-gauge needle and used for IVM.

**Animal care and use**

All animals received humane care according to Law No. 105 and Notification No. 6 and no. 22 of the Japanese Guidelines for Animal Care and Use. The experiments performed on live animals were approved by the Animal Care Committee of the National Livestock Breeding Center.

**Ovum pick-up (OPU)**

OPU was performed as reported previously by Imai et al. [3] but without follicle from approximately 28–32-month-old Holstein-Friesian cows were reared under the same feeding and environmental conditions. Aspiration of 3–6 mm follicles was performed with OPU on arbitrary days of the estrous cycle using a 7.5-MHz linear transducer with needle (Cova needle, Misawa Medical, Tokyo, Japan) connected to an ultrasound scanner (SSD-1200, Aloka, Tokyo, Japan).

**In vitro maturation (IVM)**

IVM was performed based on the method described by Imai et al. [17]. The maturation medium was 25 mM Hepes buffered TCM199 (M199, Gibco BRL, Grand Island, NY, USA) supplemented with 5% calf serum (CS, Gibco BRL). Collected oocytes were evaluated by their cumulus cell morphology, cytoplasmic color and density. COCs with complete and dense cumulus oophorus were washed twice with IVM medium and cultured for 22 h in 100-µl droplets (in groups of 20/droplet) of IVM medium covered by paraffin oil (Paraffin Liquid, Nacalai Tesque, Kyoto, Japan) in 35-mm Petri dishes (Nunclon MultiDishes, Nalge Nunc International, Roskilde, Denmark) at 38.5 C in 5% CO₂ in air with saturated humidity.

**In vitro fertilization (IVF) and culture (IVC)**

IVF was carried out as reported previously by Imai et al. [17]. Briefly, at the end of IVM, ejaculated and frozen semen of a Japanese Black bull was thawed in a 37 C water bath for 30 sec and then centrifuged in 3 ml of a 90% Percoll solution at 740 x g for 10 min. Then, the pellet was resuspended and centrifuged at 540 x g for 5 min in 6 ml of sperm washing medium composed of Brackett and Oliphant solution (BO) [18] supplemented with 10 mM hypotaurine (Sigma) and 4 U/ml heparin (Novo-Heparin Injection 1000, Aventis Pharma, Tokyo, Japan). The pellet was subsequently resuspended in sperm washing medium and BO solution supplemented with 20 mg/ml bovine serum albumin (BSA, crystallized and lyophilized, Sigma) to achieve the final concentrations of 3 x 10⁶ spermatozoa/ml, 5 mM hypotaurine, 2 U/ml heparin and 10 mg/ml BSA. Droplets of 100 µl of this suspension prepared in 35-mm plastic dishes and covered by paraffin oil served as fertilization droplets. The COCs were removed from the IVM medium, washed twice in BO supplemented with 10 mg/ml BSA, placed in fertilization droplets (20 COCs/droplet) and cultured for 6 h at 38.5 C in 5% CO₂ in air with saturated humidity. The start of IVF was considered 0 h of fertilization. IVC was performed in 100 µl drops of CR1aa medium [19] supplemented with 5% calf serum covered with paraffin oil. At the end of insemination, putative zygotes were completely denuded from cumulus cells and spermatozoa by gentle pipetting with a fine glass pipette in preincubated IVC medium. Then, 15 to 20 embryos were placed separately in culture drops. Embryos were cultured at 38.5 C in 5% CO₂ under 20% O₂ tension in air with saturated humidity.

**Measurement of relative mRNA abundance**

The expressions of 21 genes (Table 1) were analyzed in oocytes and embryos by real-time quantitative PCR (qPCR). Oocytes/embryos were completely denuded by a fine glass pipette before analysis. Pooled samples of 20 oocytes/embryos derived from each treatment group were lysed in RLT buffer (RNeasy Micro Kit, QIAGEN, Hilden, Germany) and stored at −80 C until analysis. Three biological replications were performed. Total RNA was extracted and purified from pooled samples by using an RNeasy Micro Kit (QIAGEN) according to manufacturer’s instructions. Reverse transcription was performed using a ReverTra Ace™ RT Kit (Toyobo, Tokyo, Japan) by following the manufacturer’s protocols. The normality of each cDNA sample was verified by comparing glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression to those of positive and negative controls by PCR and gel electrophoresis. Quantitative mRNA expression analysis in samples was performed by qPCR at the 1/20 dilution using LightCycler® 480 SYBR Green 1 Master mix (Roche Applied Science, Penzberg, Germany) according to standard protocols. The PCR conditions were 95 C for
<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Accession no.</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product size (bp)</th>
</tr>
</thead>
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<td>Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)</td>
<td>Glycolysis</td>
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<td>TGAGCTTGACAAAGTGGTCG</td>
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<td>Glucose-6-phosphate dehydrogenase (G6PDH)</td>
<td>Pentose phosphate pathway</td>
<td>AY862878</td>
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<td>TCTTTGCCCAGGTAGTGGTCG</td>
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<td>Cyclin B1 (CCNB1)*</td>
<td>Cell cycle</td>
<td>NM_001045872</td>
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<td>AGGCGGACCCCAAGCTAAAAT</td>
<td>155</td>
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<tr>
<td>Manganese superoxide dismutase (MnSOD)</td>
<td>Antioxidative defense</td>
<td>L2092</td>
<td>GGAAGCCATCAAACGTGACT</td>
<td>AGCAGGGGGATAAGACCTGT</td>
<td>185</td>
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<tr>
<td>Octamer-4 (OCT4)</td>
<td>Pluripotency</td>
<td>NM_174580</td>
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<td>AATTCCTACGTTGGAGTTG</td>
<td>68</td>
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<tr>
<td>SRY (sex determining region Y)-box 2 (SOX2)</td>
<td>Pluripotency</td>
<td>NM_0010145463</td>
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<td>CAGATATTCCATGTTGGTTTTCAT</td>
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<tr>
<td>Connexin 43 (CX43)</td>
<td>Gap junctions</td>
<td>J05535</td>
<td>TGTTAGGAGATGGAGTTG</td>
<td>GGGTGTGGGAGAAAAAGAA</td>
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<td>Heat shock 70 kDa protein (HSP70)</td>
<td>Heat/cold stress protection</td>
<td>U09861</td>
<td>TGGGGGACAGTCGGAGAAC</td>
<td>TCCGTTGGAGTGTGGA</td>
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<tr>
<td>Glucose transporter 8 (GLUT8)</td>
<td>Glucose uptake</td>
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<td>Poly(A) polymerase (PAP)</td>
<td>Pre-mRNA polyadenylation</td>
<td>X63436</td>
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<td>TGGAGTCTTGGTGGTGTAC</td>
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<td>Growth differentiation factor-9 (GDF9)</td>
<td>Follicle development</td>
<td>AF307092</td>
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<td>AACATTTGCGAAGGGAGAG</td>
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<td>Cytochrome c oxidase 1 (COX1)</td>
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<td>GCCGAGATGCCAGATGFT</td>
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<td>Na+/K+ transporting ATPase, alpha 1 (ATP1A1)</td>
<td>Na+ and K+ homeostasis</td>
<td>BC125664</td>
<td>ACCTGTGGGACATCGGATGAC</td>
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<td>Proteolysis, cell cycle</td>
<td>BCI20949</td>
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<td>ACAAGGCGGGAGAGTAGTGTC</td>
<td>282</td>
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<td>Actin, beta (ACTB)</td>
<td>Cytoskeleton</td>
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<td>CACCATTCACCCTGCAGTCC</td>
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<tr>
<td>Histone (H2A)</td>
<td>Chromatin structure</td>
<td>NM_174809</td>
<td>ACAGCCTGTTACAGTGTGG</td>
<td>GCGAAGAATTGGGGTGGG</td>
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Aster denotes endogenous reference gene.
5 min and 45 cycles of 95°C for 10 sec, 60°C for 5 sec and 72°C for 10 sec. The quantification of transcripts in samples was performed using a LightCycler® 480 Instrument (Roche Applied Science). In each sample, cDNA levels of each gene were calculated directly by LightCycler® 480 software by comparing measured values to standard curves prepared by measurement of pooled standards diluted 1, 10 and 100 times. The quantities of the transcripts for each gene were normalized to an endogeneous reference transcript, CCNB1, that was found in preliminary results to be the most stably expressed irrespective of oocyte source and meiotic stage. For each experimental sample, the amounts of mRNA of each transcript and CCNB1 were determined from the appropriate standard curve. Subsequently, the quantity of each transcript was divided by that of CCNB1 to obtain a normalized value for each transcript.

When immature slaughterhouse-derived oocytes were compared with immature OPU oocytes, slaughterhouse-derived oocytes considered as the control were assigned a value of 1. When immature oocytes were compared with in vitro matured MII oocytes, immature samples considered as the control were assigned a value of 1. When early and late cleaving embryos were compared, early cleaving samples considered as the control were assigned a value of 1. Target values were compared with that of the control in each replication.

**Experimental design**

**Experiment 1:** This experiment was conducted to study the effect of oocyte source and IVM on mRNA abundance in oocytes. In brief, immature oocytes were collected by OPU from live donors (OPU group) or by aspiration of follicles from the ovaries of slaughtered cows stored at 15°C overnight (stored group) on different days. In each treatment group, 20 oocytes were denuded and sampled for mRNA expression analysis immediately after collection. According to preliminary results in each treatment group, such oocytes are at the germinal vesicle (GV) stage. The rest of the oocytes were in vitro matured. At the end of the IVM period, oocytes were denuded and those with the first polar body (presumptive MII stage) were sampled for mRNA expression analysis as described above. Three trials of three pools (each containing 20 oocytes) were performed in each treatment group.

**Experiment 2:** This experiment was conducted to investigate if a reduced ATP1A1 level in late cleaving embryos is carried over to the later stages. After IVM and IVF of slaughterhouse-derived oocytes, early and late cleaving embryos were separated according to Experiment 2. Expression levels of ATP1A1 in embryos were compared between early and late cleavage groups after first cleavage (2-cell stage), at 72 hpi (8-cell stage) and at 168 hpi (blastocyst stage).

**Statistical analysis**

All data are expressed as means ± SEM. Relative mRNA expression results were compared by one-way ANOVA using the KyPlot package (Ver. 4.0, KyensLab, Tokyo, Japan). Percentage data for embryo development were arc sine transformed before analysis. Differences of P<0.05 were considered to be significant.

**Results**

**Effect of ovary storage on relative mRNA abundance in immature oocytes**

The relative mRNA abundances of 20 genes in immature oocytes obtained from different sources are shown in Fig. 1. The expression of GAPDH, OCT4, HSP70, GLUT8, ATP1A1, DYNLL1 and DYNC1I1 genes showed significantly higher levels in OPU oocytes than those in slaughterhouse-derived oocytes.

**Effect of IVM on relative mRNA abundance in oocytes**

The relative mRNA abundances of genes in oocytes obtained from different sources before and after IVM are shown in Fig. 2. In slaughterhouse oocytes, the expression of GLUT8, DGF9, CTNNB1 and PMSB1 showed significantly reduced levels (P<0.05) compared with the relative expression levels before IVM (Fig. 2A). The relative mRNA abundance of the other genes did not differ significantly between immature and IVM oocytes.

When OPU oocytes were subjected to IVM (Fig. 2B), similar to slaughterhouse oocytes, the expression levels of GLUT8, DGF9, CTNNB1 and PMSB1 genes were significantly reduced (P<0.01) after IVM. Furthermore, the expression of GAPDH and MnSOD also showed reduced (P<0.01) levels in IVM oocytes compared with those before IVM.

**Developmental competence and mRNA expression in early and late cleaving embryos obtained from slaughterhouse oocytes**

When IVM/IVF oocytes were separated according to the timing of first cleavage, it was found that oocytes showing early cleavage showed significantly higher in vitro developmental competence to the blastocyst stage than those showing a late cleavage (Fig. 3A). When mRNA expression levels were measured in 2-cell embryos, the ATP1A1 gene showed significantly higher expression in the early cleavage groups compared with late cleavage groups (Fig. 3B). The relative mRNA abundance of the other genes did not differ between early and late cleaving 2-cell embryos (Fig. 3B).

**Expression levels of ATP1A1 in early and late cleaving embryos at different stages of development**

When early and late cleaving embryos were cultured separately,
Significantly reduced expression of *ATP1A1* was observed in the late cleaving group at the 2-cell stage and also at the 8-cell stage compared with the early cleaving group. However, blastocysts obtained from the early and late cleaving groups showed similar levels of *ATP1A1* expression (Fig. 4).

**Fig. 1.** Relative gene expression in immature bovine oocytes of different sources after oocyte retrieval. Treatment groups: stored = oocytes collected from ovaries of slaughtered animals by aspiration after storage at 15°C for overnight; OPU = oocytes collected by OPU without follicle superstimulation. Asterisks denote significant differences at P<0.05.

**Fig. 2.** Relative gene expression in immature and *in vitro* matured bovine oocytes of different sources. A: oocytes collected from ovaries of slaughtered animals by aspiration after storage at 15°C for overnight; B: oocytes collected by OPU without follicle superstimulation. Treatment groups: 0 h IVM (immature) = presumptive GV-stage oocytes before IVM; 22 h IVM (MII) = presumptive MII-stage oocytes at 22 h of IVM. Asterisks (*) and (**) denote significant differences at P<0.05 and P<0.01, respectively.

**Fig. 3.** *In vitro* development (A) and relative mRNA levels (B) in 2-cell stage IVP embryos generated from slaughterhouse oocytes showing early (22–25 hpi) or late (27–30 hpi) first cleavage. The asterisk denotes a significant difference at the P<0.05 level. Total numbers of embryos used for embryo culture (n) are given in parentheses.
Oxidative stress caused by atmospheric tensions of O2 also exerts induced by cryopreservation reduces mRNA levels in oocytes [29]. Recent research has revealed that extreme cold stress and the lack of nutrients caused by removing the ovary from the stresses on oocytes such as low temperature, high oxygen levels and cold storage of ovaries and IVM exerts different kinds of discrepancies might be attributed to differences in protein supplementation (i.e., BSA) between studies, which are known to affect mRNA expression in oocytes [27, 28].

**Discussion**

**Effect of ovary storage and IVM on mRNA levels in oocytes**

Our results have revealed that removal and overnight storage of bovine ovaries at 15 C significantly reduce the mRNA levels of several genes such as GAPDH, OCT4, HSP70, GLUT8, ATP1A1, DYNILL1 and DYNC11/1 in immature oocytes. After ovary storage, further reductions in the expression levels of oocyte mRNA were observed during subsequent IVM culture in accordance with previous studies on bovine and murine oocytes [21–26]. In our study, the expression of GLUT8, GDF9, CTNNB1 and PMSB1 was significantly decreased during IVM irrespective of oocyte source. Other genes such as GAPDH and MnSOD showed reduced expression in mature oocytes only in the OPU group. Further research comparing OPU-derived immature and in vivo matured oocytes will be needed to clarify if the reduction of maternal mRNA during IVM of these genes is a normal process or is caused by the imperfection of the IVM system. In contrast with our results, the expressions of DYNLL1 and DYNC11/1 were also found to be reduced in bovine oocytes during IVM in a previous study [21]. The reason for this discrepancy might be attributed to differences in protein supplementation (i.e., BSA vs. serum) between studies, which are known to affect mRNA expression in oocytes [27, 28].

The exact mechanism that causes the reduction of mRNA levels in oocytes during ovary storage and culture is not clear. Removal and cold storage of ovaries and IVM exerts different kinds of stresses on oocytes such as low temperature, high oxygen levels and the lack of nutrients caused by removing the ovary from the blood flow. Recent research has revealed that extreme cold stress induced by cryopreservation reduces mRNA levels in oocytes [29]. Oxidative stress caused by atmospheric tensions of O2 also exerts various effects on animal cells including alterations in metabolic processes at the molecular level [30]. Furthermore, previous reports have revealed the severe reduction of glucose concentration in follicle fluid during ovary storage, which was associated with decreased oocyte developmental competence [31, 32]. In fact, removal and storage prevent blood flow and thus energy supplies in organs, and therefore, maintaining normal ATP levels in tissues is considered to have basic importance for organ preservation [33]. One might think that reduced levels of transcripts in stored oocytes might be related to repressed metabolism. Nevertheless, our additional experiments showed that blocking ATP synthesis in immature bovine oocytes with 10 μM oligomycin for 1 h did not affect the expression of GAPDH, GLUT8, HSP70 or ATP1A1 (data not shown). This result suggests that repressed metabolism in oocytes during ovary storage might not be the reason for reduced expression of these genes. The exact mechanism that lies behind the reduction of mRNA levels in oocytes obtained from stored ovaries requires further clarification. It must be noted that data on mRNA levels in oocytes from fresh slaughterhouse ovaries (lacking in the present study) may be required to understand the effects of ovary storage on oocytes at the molecular level. Furthermore, possible differences in the feeding conditions, lactation stage and body condition between slaughtered animals and those subjected to OPU could have also affected the results.

**Possible consequences of mRNA reduction in oocytes**

A reduced mRNA level suggests altered cellular processes, which may affect viability and developmental competence in IVM oocytes derived from stored ovaries. For instance, the expression of genes related to metabolism such as GAPDH and GLUT8 and stress such as HSP70 were reduced, which may suggest repression in metabolic processes and cellular defense mechanisms in oocytes from stored ovaries. GAPDH encodes glyceraldehyde 3-phosphate dehydrogenase, a regulator of glucose-based energy production, and this gene is often used as an endogenous reference to demonstrate relative gene expression levels. Therefore, it is important to point out that GAPDH might not be the optimum choice for an internal reference gene when mRNA samples are taken from ovaries stored at low temperatures.

In our experiments, nuclear maturation of IVM oocytes to the MII stage was found to be similar between slaughterhouse and OPU-derived oocytes (data not shown). Thus cellular processes altered by ovary storage may not affect meiotic competence of oocytes. On the other hand previous reports have demonstrated the detrimental effect of ovary storage on oocyte developmental competence [6]. To understand if compromised competence of oocytes obtained from stored ovaries could be potentially attributed to altered mRNA levels, we attempted to clarify in a series of experiments if maternal mRNA levels in slaughterhouse-derived cleavage stage embryos correlate with their developmental ability to the blastocyst stage.

**Importance of maternal mRNA levels in cleavage stage embryos for developmental competence to the blastocyst stage**

Many of the genes examined in the present study such as those of E-cadherin (CDH1), β-catenin (CTNNB1), aquaporins (e.g., AQP3) or Na+/K-ATPase (ATP1A1) are known to play roles in blastocyst formation in mammals [34]. A previous study on β-catenin-

![Fig. 4.](image-url)
deficient mice has demonstrated the importance of maternally inherited \( \beta \)-catenin mRNA for embryo development even after zygotic genome activation [35]. These reports and our results on reduction of mRNA levels during oocyte collection and IVM have focused our interest on the importance of maternal mRNA levels in embryos obtained from slaughterhouse oocytes before embryo genome activation for the developmental competence of embryos beyond the 8-cell stage. Such knowledge is scarce in cattle because technological difficulties in producing knockout or transgenic animals beyond the 8-cell stage. Such knowledge is scarce in cattle because technological difficulties in producing knockout or transgenic animals in this species. Therefore, in this study, mRNA expression in IVF embryos at the 2-cell stage (before genomic activation) with high and reduced developmental competence was compared. Developmental competence was determined according to the timing of the first embryonic cleavage, which is considered to be a potent marker for the competence of mammalian embryos [36]. Our present results have confirmed that selection of newly cleaved embryos at 22–25 hpi and 27–30 hpi is an effective way to separate 2-cell stage embryos with high and low developmental competence, respectively.

Previous studies have revealed differences in gene expressions between early and late cleaving bovine embryos [11–13, 37]. In the present study, only the Na/K ATPase encoding \( ATP1A1 \) showed a correlation between its expression levels in 2-cell stage embryos and their developmental competence to the blastocyst stage. In developing mammalian embryos, Na/K ATPase plays an important role during blastulation by maintaining an ionic gradient across the trophoderm promoting the osmotic accumulation of water across the epithelium [34]. According to the present results, \( ATP1A1 \) expression was reduced in late-cleaving embryos already at the 2-cell stage and even at the 8-cell stage compared with the early cleaving group. Low expression of \( ATP1A1 \) until the beginning of embryonic genome activation in the late cleavage group was associated with a low blastocyst formation rate, suggesting the possibility that maternal transcripts of this gene may be important for successful blastocyst formation. On the other hand, maternally derived \( ATP1A1 \) mRNA might not determine embryo development beyond the blastocyst stage, since blastocyst derived from the early and late cleaving groups in the present study showed similar expression of \( ATP1A1 \) and such blastocyst do not differ in cell numbers and allocations of inner cell mass and trophoderm cells (unpublished results).

Although maternal mRNA levels of several other genes such as \( HSP70 \), \( GLUT8 \), \( GDF9 \) and \( PMSB1 \) were also found to drop in oocytes during ovary storage and IVM, their levels at the 2-cell stage did not correlate with developmental competence. This might either suggest that 1) maternal mRNA levels of these genes do not have a direct effect on blastocyst formation or 2) the reduction of mRNA did not drop below an assumed threshold level that would limit developmental competence of oocytes to the blastocyst stage.

In a previous study, Dode et al. [12] compared embryos that cleaved between 24–32 hpi to those that cleaved between 36–44 hpi. On the other hand, in the present study, we defined early cleavage as occurring at 22–25 hpi and late cleavage as occurring at 27–30 hpi based on our previous results using time-lapse cinematography under the same conditions [20]. In our IVP system, the number of embryos showing the first cleavage after 30 hpi was very scarce, and such oocytes were often found to have resulted from abnormal cleavage such as fragmentation.

Removal and storage of ovaries reduced maternal mRNA levels of \( GAPDH \), \( GLUT \), \( HSP70 \), and \( ATP1A1 \). During IVM, further reductions in mRNA levels of \( GLUT8 \), \( GDF9 \) and \( PMSB1 \) were observed irrespective of ovary storage. Among these genes, maternal mRNA levels of \( ATP1A1 \) in early stage slaughterhouse-derived embryos correlated with their ability to develop to the blastocyst stage. Therefore, ovary storage might affect oocyte developmental competence by reducing intracellular mRNA levels of \( ATP1A1 \). During early development of slaughterhouse-derived embryos, reduced levels of maternal \( ATP1A1 \) mRNA may either directly limit blastocyst formation or at least reflect developmental competence of early embryos to the blastocyst stage. Therefore, we consider this gene to be a possible marker for early developmental competence in early bovine embryos.

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